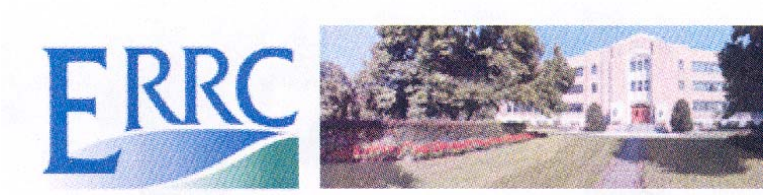

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Growth and Heat Resistance Kinetic Variation Among Various Isolates of *Salmonella* and its Application to Risk Assessment

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The abilities of cells of a particular type of bacteria to leave lag phase and begin the process of dividing or surviving heat treatment can depend on the serotypes or strains of the bacteria. This article reports an investigation of serotype-specific differences in growth and heat resistance kinetics of clinical and food isolates of *Salmonella*. Growth kinetics at 19°C and 37°C were examined in brain heart infusion broth and heat resistance kinetics for 60°C were examined in beef gravy using a submerged coil heating apparatus. Estimates of the parameters of the growth curves suggests a small between-serotype variance of the growth kinetics. However, for inactivation, the results suggest a significant between-serotype effect on the asymptotic D-values, with an estimated between-serotype CV of about 20%. In microbial risk assessment, predictive microbiology is used to estimate growth and inactivation of pathogens. Often the data used for estimating the growth or inactivation kinetics are based on measurements on a cocktail—a mixture of approximately equal proportions of several serotypes or strains of the pathogen being studied. The expected growth or inactivation rates derived from data using cocktails are biased, reflecting the characteristics of the fastest growing or most heat resistant serotype of the cocktail. In this article, an adjustment to decrease this possible bias in a risk assessment is offered. The article also presents discussion of the effect on estimating growth when stochastic assumptions are incorporated in the model. In particular, equations describing the variation of relative growth are derived, accounting for the stochastic variations of the division of cells. For small numbers of cells, the expected value of the relative growth is not an appropriate "representative" value for actual relative growths that might occur.

KEY WORDS: Survival curves; growth curves; variance components; nonlinear; stochastic

1. INTRODUCTION

Gram-negative pathogens such as *Salmonella* and *Escherichia coli* are a continuing concern to the food industry because they can survive in foods under a myriad of conditions. To design processes for ensuring microbiological safety, studies of growth or inactivation of the pathogen of concern are conducted assuming conditions similar to those that occur during the preparation and processing of raw agricultural commodities into ready-to-eat processed foods. Such studies involve a careful review of the published literature regarding the incidence of food-borne illness, mortality, and recalls associated with the product. It is not possible to study the growth and inactivation kinetics of all the serotypes or strains that can contaminate

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food and thus cause a potential risk if consumed. Consequently, a number of serotypes are selected and composite cultures (a "cocktail") of them are studied under different conditions. Because the asymptotes of the derived curves are thought to be describing the asymptotic kinetics for the fastest growing or most resistant of the selected serotypes in the cocktail for a given condition, the derived parameters used to describe the growth or survival curves from data obtained from cocktails are thought to be conservative regarding the possible growth or inactivation of the organisms in a risk assessment application. The selected serotypes for a cocktail are usually the ones thought to have the highest growth rates and the most heat resistance among serotypes that are found in the food matrix of interest, excluding those with atypical growth characteristics or unusually high heat resistance, such as *Salmonella senftenberg* 775W,[1] that are not found in the food matrix of interest. The consequence for a risk assessment is that, although the measured values of the kinetic parameters are presumed to be conservative, the inherent between-serotype variability of the kinetic parameters is not accounted for in the risk assessment. Basically, the expected values of the estimated survival or growth curves would be biased with respect to the true "average" curves for the population of *Salmonella* spp serotypes, and the between-serotype variances of the curves would not be known. This article discusses possible procedures of adjustment that would account for the bias that is created by using a cocktail.

A natural consideration for a risk assessment is the relationship between the cell's ability to grow and multiply and the cell's ability to survive adverse environmental effects, such as heat. A more heat resistant cell, associated with a higher D-value—the time to reduce the population by 90% at a given temperature—might also be a cell that has a faster growth rate. Or, alternatively, the mechanism that enhances a cell's ability to resist and stay viable may hinder or not contribute to its ability to grow and multiply. Thus, modeling the risk associated with pathogens in foods entails identifying a multivariate distribution characterizing the variances of the growth and lethality kinetic parameters and the correlations among them.

The usual outputs of predictive microbiology are estimates of growth and survival curves that represent, in some statistical sense, the expected values of the curves. An important issue for risk assessment is the application of these curves to a small number of cells. The ability of cells to survive or to "leave" the lag phase and begin the process of dividing varies among cells of an otherwise homogeneous populations of cells. This inherent variability, particularly when there are a small number of cells, could be important in a risk assessment for determining the distribution of the number of cells as a function of time.

Accordingly, the aim of the present work is to determine and compare the growth characteristics and heat resistance of various *Salmonella* serotypes and to discuss possible applications and associated concerns for risk assessment. There have been a few studies published on the growth and inactivation kinetic parameters of different serotypes of pathogens, and many of these have shown, to varying degrees, significant serotype variability.[2–8] In these studies, the components of variances that would be used in a risk assessment, such as the between-serotype variance component, are not reported. In this article, estimates of variance components are made.

In addition, this article presents a short discussion concerning the variability of growth of a small population of cells. Equations that are developed in predictive microbiology provide the predictions of the expected amount of relative growth, or actually the logarithm of the relative growth, for fixed conditions, often temperature. However, for small numbers of cells the expected value may not be an appropriate value for use in a risk assessment: it is possible that for small numbers of cells there are significant probabilities of no growth or large amounts of growth. Equations to determine these probabilities are developed from which the probabilities of no growth and of large amounts of growth are computed using the parameters of the estimated growth models of *Salmonella* derived in this article.

2. MATERIALS AND METHODS

The procedures used for measuring levels of bacteria in samples over time follow standard procedures that have been used in previous studies.[9,10] Since the purpose of this article is to present a discussion of risk assessment issues, a short accounting of the procedures used is given. For more details, interested readers can examine the articles referred to above.

2.1. Bacterial Serotypes

Salmonella serotypes isolated from raw processed beef, pork, chicken, and turkey, as well as human clinical isolates, were used in this study. The

Table I. *Salmonella* Serotypes

Serotype/Strain	Isolate Designation	Source	Origin
Salmonella Kentucky*	062	FSIS (1)	Chicken
S. Thompson	120	FSIS (8)	Chicken
S. Enteritidis, phage type 13a*	H3527	CDC (10)	Clinical
S. Enteritidis, phage type 4	H3502	CDC (11)	Clinical
S. Typhimurium, phage type DT104	H3380	CDC (14)	Clinical
S. Hadar*	MF60404	FSIS (20)	Turkey
S. Copenhagen*	8457	NVS Labs (21)	Pork
S. Montevideo	051	FSIS (24)	Beef
S. Typhimurium*	026	FSIS (25)	Beef
S. Heidelberg	F5038BG1	CDC (40)	Stuffed ham/chad slicer

*Serotypes for which both growth and survival curves were estimated.

information about these serotypes is given in Table I. These serotypes were stored at –70°C in a mixture (85:15; v/v) of Tryptic Soy Broth (TSB; Difco; Detroit, MI) and glycerol (Sigma Chemical Co., St. Louis, MO).

2.2. Preparation of Test Cultures

To prepare the cultures, vials were partially thawed at room temperature and 1.0 ml of the culture was transferred to 10 ml of brain heart infusion broth (BHI; Difco) in 50-ml tubes and incubated for 24 hours at 37°C. This culture was not used in growth or heating studies as it contained freeze-damaged cells. A working culture for use in growth and heating studies was prepared by transferring 0.1 ml of each culture to 10-ml tubes of BHI and incubating aerobically for 24 hours at 37°C. These cultures were maintained in BHI for two weeks at 4°C. A new series of cultures was initiated from the frozen stock on a biweekly basis.

A day before the experiment, the inocula for conducting the growth and heating studies were prepared by transferring 0.1 ml of each culture to 10-ml tubes of BHI, and incubating aerobically for 18 hours at 37°C to provide late stationary phase cells. On the day of the experiment, each culture was centrifuged (5,000 × g, 15 min, 4°C), the pellet was washed twice in 0.1% peptone water (wt/vol) and finally suspended in peptone water to a target level of 8–9 $\log_{10}$ cfu/ml. The population densities in each cell suspension were enumerated by spiral plating (Model D; Spiral Biotech, Bethesda, MD) the appropriate dilutions (in 0.1% peptone water), in duplicate, on Tryptic soy agar (TSA; Difco) plate and incubating at 37°C for 48 hours.

2.3. Growth Experiment

Brain heart infusion broth (BHIB, 100 ml) in 250-ml flasks were sterilized for 15 minutes at 121°C. Each flask was inoculated with 0.1 ml of the diluted inoculum of an 18-hour culture of *Salmonella* to yield a starting level of approximately 2–3 log cfu/ml, and then incubated at 19 or 37° on a model G-26 rotary shaker (120 rpm). At intervals appropriate for the temperature, samples were withdrawn for enumerating the bacteria by serial dilutions (in peptone water), surface plating with a spiral plater onto TSA as mentioned above. Two replications were performed for each temperature.

2.4. Thermal Inactivation Procedure

The formulation of the model beef gravy used in the present study as heating menstruum was: 1.5% protease peptone, 5.0% beef extract, 0.5% yeast extract, and 1.7% soluble starch. All ingredients were obtained from Difco Laboratories (Detroit, MI). The gravy was sterilized by autoclaving prior to use. Beef gravy (10 ml) was inoculated with 0.1 ml of the diluted inoculum of selected *Salmonella* or *E. coli* isolates to obtain a final concentration of approximately 7–8 log cfu/ml. Thereafter, the gravy suspensions were heated at 60°C using a submerged coil heating apparatus.[11] The submerged coil heating apparatus is comprised of a stainless steel coil fully submerged in a thermostatically controlled water bath, which allows microbial suspensions to be heated, with a short time to achieve temperature equilibrium within the range of 20–90°C. During the heating procedure, samples (0.2 ml) were removed at predetermined time intervals. Where low cell numbers were expected, 0.6 ml aliquots were removed. Samples were cooled rapidly in ice slurry.

2.5. Enumeration of Surviving Bacteria

Decimal serial dilutions were prepared in peptone water and appropriate dilutions surface plated in duplicate on TSA, supplemented with 0.6% yeast extract and 1% sodium pyruvate, using a spiral plater. Samples not inoculated with *Salmonella* were plated as controls. Also, 0.1 and 1.0 ml of undiluted suspension were surface plated, where relevant. All plates were incubated at 30°C for at least 48 hours prior to counting colonies. For each replicate experiment, an average cfu/g of duplicate platings at each sampling point were used in the statistical analysis.

2.6. Statistical Methods

2.6.1. Growth Curves

Our main concern is to determine the growth curve during the lag and exponential phases of growth. Lag times before the cells are ready to divide depend on many environmental factors and possibly the initial levels.[12] However, for *Salmonella*, information on the effects of initial levels on lag phase duration and growth rates is not available to us. Thus, it is assumed that relative growth curves are independent of the initial levels. In these studies, the initial levels were about 2–3 $\log_{10}$.

The equation for predicting growth is derived by assuming that cell division involves two stages:[13,14] (1) an original cell (O-cell) in stationary phase passing through an initial state, entering into a similar physiological state as that of a new born cell (D-cell) and (2) a D-cell dividing into two, newborn D-cells. The equation used to estimate the growth curves at a given temperature is:

$$\log_{10}(E(r(t))) = \log_{10}(\mu e^{-\lambda t} + \lambda e^{\mu t}) - \log_{10}(\mu + \lambda) \quad (1)$$

where $r(t)$ is the observed relative growth at time t, λ is the exponential rate that original cells leave lag, and μ is the exponential growth rate of cells no longer in lag. This equation corresponds to the equation developed by Baranyi[13:Eq. 23] for estimating growth curves for cells in lag and exponential phases.

The mathematical lag time, a characteristic of the population growth curve, is defined as the intersection of the horizontal line, $y = n_0$ (n_0 is the log of the initial level), and the asymptotic line of the curve as t approaches infinity.[13,15] The mathematical lag time, *lagGrowth*, is thus:

$$lagGrowth = \frac{\ln(1 + \mu/\lambda)}{\mu}. \quad (2)$$

Following customary practice, the exponential growth rate in $\log_{10}$ units, egr_{10}, is reported. Thus:

$$egr_{10} = \mu/\ln(10). \quad (3)$$

A statistic of interest[13,16] for modeling purposes is the product of the exponential growth rate, egr_{10}, and the *lagGrowth*:

$$\omega = \ln(1 + \mu/\lambda)/\ln(10). \quad (4)$$

The value of ω has been shown to be relatively less variable[16] over different temperatures or other different environmental conditions compared to the other parameters mentioned above.

2.6.2. Survival Curves

The survival curves considered can be characterized by an initial shoulder for small times, that, for large times, asymptotically approaches a straight line with slope equal to the negative of the inverse of the asymptotic D-value. Many equations for describing nonlinear survival curves have been developed,[9,17–22] some motivated by mechanistic considerations, others by just providing good empirical fits. In attempting to find a set of curves that provides a good fit to the observed data, three different functions were examined. The first equation considered[9] has two parameters: k and $w > 0$:

$$\log_{10}(E(r(t))) = -kt + \log_{10}\left(1 + \frac{k}{w}(1 - e^{-wt})\right) \quad (5)$$

where $r(t)$ is the observed relative decline. As $t \to \infty$, the derivative of $\log_{10}(E(r(t))) \to -k$, and as $t \to 0^+$, the derivative approaches 0 so that Equation (5) describes a survival curve with an asymptotic D-value and curved "shoulders" with initial slope equal to 0. However, most of the observed data did not follow a pattern that would suggest a survival curve with this latter property. Thus an adjustment to Equation (5), which permits nonzero initial slope, was made by setting the coefficient of $(1 - e^{-wt})/w$ in Equation (5) to an arbitrary constant. This model is referred to as the 1-stage (modified) model.

A second model considered,

$$\log_{10}(E(r(t))) = -kt - a(e^{-bt} - 1). \quad (6)$$

This is a modification of an equation developed by Han[17] for heat activation of spores, and further described by Sharpe and Bektash.[18] In those papers, the survival curves considered were convex, and there was a relationship of the parameter values such that

second derivative of $\log_{10}(E(r(t)))$ was positive for all t. For our application, if it is assumed that if a and $b \geq 0$, then, for all t, the second derivative of $\log_{10}(E(r(t)))$ is negative and as $t \to \infty$, the derivative $\to -k$. Furthermore, as $t \to 0^+$, the derivative $\to -k + ab$ and thus can be positive, representing an initial increase in the cell population before cell inactivation begins. This function provides the capability to fit curves to data for which it appears that the derivative at $t = 0$ is not zero.

The final model considered also has this property and is motivated by using the logistic function as follows:

$$\log_{10}(E(r(t))) = -kt - \log_{10}(1 + e^{-b(t-a)}) + \log_{10}(1 + e^{ba}). \quad (7)$$

The logistic function has been used for fitting survival curves[20] and is a flexible function that provides good capability of fitting curves to data sets that display different relations.

For determining the values for the parameters of the regressions, OLS nonlinear regression routines were used from SAS®—PC release 8.00, PROC NLIN,[23] where the observed levels of cfu/ml were used as the dependent variable. The fitted survival curve equations thus include the numbers of cells at time equal to 0 as parameters. Parameter values were estimated using the default secant method (DUD procedure), which is a Gauss-Newton procedure, except the partial derivatives are estimated numerically from the iterations. For computing root mean square errors (RMSE) for the models, the sum of squares of the residuals was divided by $n - p$ where n is the number of data observations and p is the number of parameters in the model.

2.6.3. *Statistical Analysis*

An analysis of variances (AOV) is used to estimate significance of factors and variance components. For our purposes, the (mixed) model for expressing the result, y, the dependent variable: the natural logarithm of either the exponential growth rate; the product of the exponential growth rate and the *lagGrowth*, ω (Equation (4)); or the lethality inactivation rate (D-value), as a function of the s^{th} serotype, e^{th} replicate, k^{th} trial within the e^{th} replicate for a specified condition, C, can be written as

$$y_{s,e,C,k} = \mu_C + \alpha_s + \alpha_{s,C} + \alpha_e + \alpha_{s,e} + \alpha_{w(e,s,C,k)} \quad (8)$$

where μ_C is the mean for the population of serotypes for the given condition, C, of the experimental scenario; α_s and $\alpha_{s,C}$ are the effect of the s^{th} serotype and interaction with the condition of the experiments; α_e is the effect for the e^{th} replicate; $\alpha_{s,e}$ is the interaction of the s^{th} serotype and e^{th} replicate; and $\alpha_{w(e,s,C,k)}$ is the "residual" error that would include the measurement error arising from the k^{th} trial within a replicate and possible differential effects of the experiment on the s^{th} serotype, or higher-order interaction effects of the experimental condition, serotype, and replicate. The effects are assumed to have means equal to zero and variances equal to σ_z^2, where z ranges over the subscripts of the above effects, and are assumed to be uncorrelated. The magnitudes of the effects, and thus the variances of them, may depend upon the condition C of the experiments. Generally, it is assumed that higher-order interaction effects are negligible so that, for example, repetitions within a replicate would permit an estimate of $\sigma_{w(e,s,C,k)}$. However, this assumption may not be innocuous.

An interpretation of the variance components: σ_s^2 and σ_{sC}^2, is that $\sigma_s^2/(\sigma_s^2 + \sigma_{sC}^2)$ represents the (intraclass) correlation of serotype effects for different conditions; if the correlation were close to 1, then the interaction would be close to 0; if the correlation were close to 0, then σ_s^2 would be close to 0 in comparison to σ_{sC}^2—meaning that over the different conditions, there is not a pronounced average serotype effect, relative to the serotype effect for (within) a given condition. This latter possibility of a pronounced interaction seems unlikely; for example, it seems unlikely that a given serotype grows faster or has greater heat resistance at one temperature and grows slower or has lesser heat resistance at a different temperature, relative to the other serotypes. If the thermal death curves (TDC) were linear then $E(\Delta y_S) = b_S \Delta T$, where b_S is the slope the slope for the S^{th} serotype. For two given temperatures, T_j, $j = 1, 2$, the variance of b_S is $2\sigma_{sC}^2/(T_1 - T_2)^2$, so that if there is no interaction ($\sigma_{sC}^2 = 0$), then there is a common z-value for the population of serotypes.

The term "replicate" refers to what we consider an independent trial or a set of trials, but performed by the same analyst, out of necessity, in different periods of time with different preparatory materials. Within a replicate, there are experiments conducted with different serotypes or different temperatures, as the case may be. For the studies presented in this article, replicates including different conditions are independent, so that the interaction term $\alpha_{e,C}$ can be assumed to be 0. The variance of results obtained from repetitions

within replicates is assumed to provide an unbiased estimate of $\sigma_{w(e,s,C,k)}$. Our primary goal in this analysis is to estimate σ_s, and to consider $\sigma_{s,C}$, where possible. Our goal is achieved, at least in an unbiased way, only to the degree that what we have called replicates are actually true independent realizations; if analyst effects were significant, our goal would not be achieved.

The results of the AOV will be reported as coefficients of variation of the exponential growth rates or D-values, as the case may be, using the approximation, $CV(x) \approx 100\text{std}(\ln(x))$, where x is a random variable. If the underlying distribution is log-normal, then, for CVs less than 20%, the percent error of this approximation is less than or equal to 1%; for example, an estimate of CV = 20% is obtained when the true CV is 20.2%. The actual variance components are estimated using SAS®—PC release 8.00, PROC VARCOMP, the default MIVQUE0 option.[23]

3. RESULTS

3.1. Growth Curves

Figs. 1 and 2 present graphs of the observed data and the fitted growth curves at temperatures = 19°C and 37°C, respectively, for the different *Salmonella* serotypes. For *Salmonella* at 19°C, there were small number of observed data per replicate, thus, at this temperature, one curve was fit for each serotype.

Table II presents the estimated exponential growth rates, $egr_{10}(\log_{10}/h)$, lag times, *lagGrowth*(h), and product of the exponential growth rate and *lagGrowth*, ω, for each *Salmonella* serotype, and the averages over the serotypes together with standard errors. At 19°C and 37°C, respectively, the averages of the egr_{10}s, are 0.26 $\log_{10}$/h and 1.26 $\log_{10}$/h; the averages of the lag times are 5.3 and 2.1 hours; and the averages of ω are 1.4 and 2.8. The difference of the averages of ω is statistically significant at near the 0.01 level, based on a two-sample *t*-test. However, when considering replicates as a random factor, the difference is not statistically significant (*P*-value = 0.38).

For egr_{10} at 37°C, there is a replicate effect ($\sigma_e^2 > 0$), where the average of the estimated egr was higher for the first replicate. Accounting for this effect and using the AOV of Equation (8) with $\ln(egr_{10})$ as the dependent variable, the between-serotype variance, σ_s^2, is estimated to be negative. Assuming that $\sigma_s^2 = 0$, the residual error CV of egr_{10} is estimated to be about 16%. Considering the replicate effect as a random effect, the total CV is estimated to be about 22%. At 19°C, there was only one replicate per

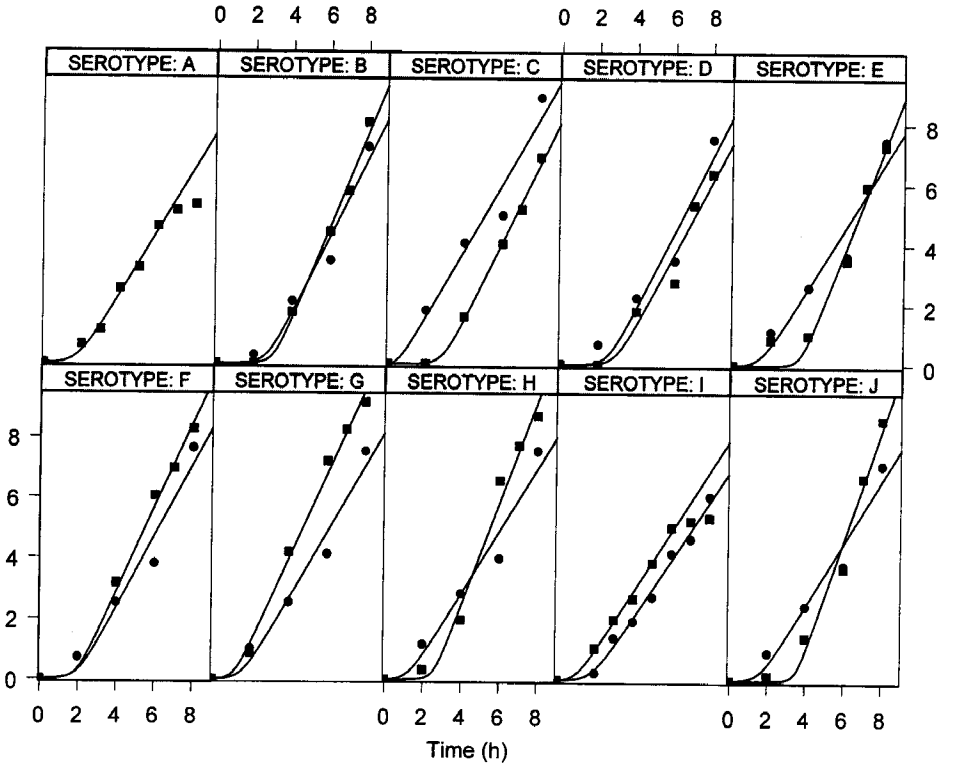

Fig. 1. Observed and fitted growth curves at 19°C for 10 serotypes of *Salmonella*, labeled A–J in order as presented in Table I.

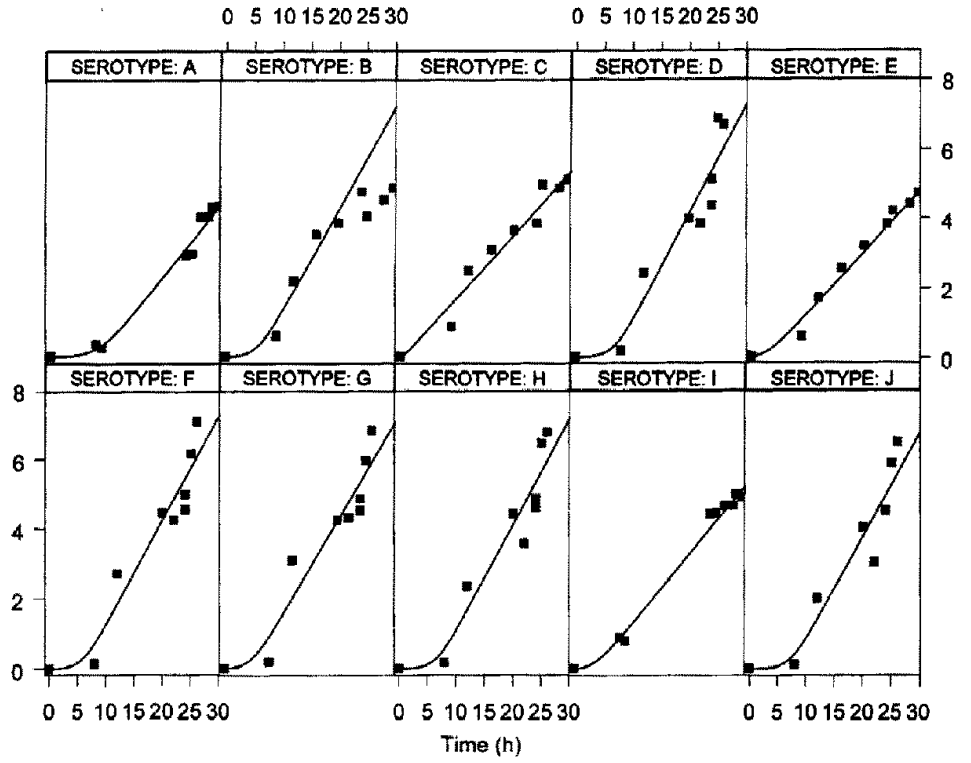

Fig. 2. Observed and fitted growth curves at 37°C for 10 serotypes of *Salmonella*, labeled A–J in order as presented in Table I. Observed points for replicate 1 indicated by ■; replicate 2 by ●.

serotype, and the (total) CV of the estimated egr_{10} is about 24%. Pooling results over both temperatures and using Equation (8), the temperature-serotype interaction variance component, σ^2_{sC}, is negative, the serotype CV, based on σ^2_s, is about 10%, and the residual CV is 16%.

For the product of the exponential growth rate, egr_{10}, and *lagGrowth*, ω, at 37°C, as with egr_{10}, the AOV indicated a statistically significant replicate effect. Accounting for this effect, using the AOV of Equation (8) with $\ln(\omega)$ as the dependent variable, the between-serotype variance, σ^2_s, is estimated to be negative. Assuming that $\sigma^2_s = 0$, the residual standard deviation, σ_r, is estimated to be about 0.57. At 19°C, there was only one replicate per serotype, and the total standard deviation of $\ln(\omega)$ is 0.93. The AOV, Equation (8), on $\ln(\omega)$ yielded a negative temperature-serotype interaction, the between-serotype standard deviation, σ_s, was estimated as 0.34, and a residual standard deviation, σ_r as 0.62. Also from the analysis of variance, the between-serotype correlation of $\ln(egr_{10})$ and $\ln(\omega)$ is estimated to be 0.79, and the correlation of the residuals is estimated to be 0.74.

Coefficient of variations of estimated generation times for 45 strains of *Salmonella* enteritidis are reported[4] for selected temperatures ranging from 7–42°C. The generation time is inversely proportional to the egr, so that the CV of the generation time equals, in approximation, the CV of the egr. The reported CV values generally decrease with increasing temperature. At 17°C and 22°C, the estimated CV values range between 10% and 15%, which is considerably smaller than the 24% estimate given above at 19°C. At 37°C, the reported CV is 3.8%, which again is considerably smaller than the 16% estimate of the replicate CV given above at 37°C. Estimates of the experimental or replication CV values were not reported in the article,[4] and thus it is not clear what the magnitude of the between-strain or serotype CV might be. However, the small estimated CV values reported in the article suggest a negligible between-strain CV for *Salmonella* enteritidis.

3.2. Survival Curves

As mentioned above, the survival curves appear to have shoulders and asymptotic D-values. For the 1-stage adjusted model, the estimates of the values for w were mostly zero, thus the actual model that was fit assumed $w = 0$; that is, the fitted function is

Serotype	19°C			37°C		
	egr $\log_{10}$/h	Lag (h)	ω $\log_{10}$	egr $\log_{10}$/h	Lag (h)	ω $\log_{10}$
A	0.204	8.701	1.774	1.048	1.752	1.836
B	0.294	5.655	1.662	1.401	2.818	3.974
C	0.181	0.642	0.116	1.202	1.700	2.159
D	0.312	6.715	2.092	1.230	2.678	3.289
E	0.177	2.845	0.503	1.336	2.512	3.678
F	0.302	5.915	1.788	1.292	1.974	2.547
G	0.280	4.906	1.373	1.252	1.377	1.693
H	0.307	6.557	2.010	1.372	1.929	2.865
I	0.198	3.787	0.751	0.978	1.474	1.435
J	0.298	7.270	2.166	1.448	2.567	4.105
Mean	0.255	5.299	1.424	1.256	2.078	2.756
SE	0.018	0.743	0.228	0.048	0.166	0.309
CV error	7.1%	14.0%	16.0%	3.8%	8.0%	11.2%

Table II. Estimates of Exponential Growth Rates, egr_{10}, ($\log_{10}$/h); Lag Time (h); and ω, the Product of Lag Times and egr_{10}, for 10 Serotypes of *Salmonella*, labeled A–J in Order as Presented in Table I, at Temperatures 19°C and 37°C

$$\log_{10}(E(r(t))) = -kt + \log_{10}(1 + bt). \quad (9)$$

Table III presents root mean square errors (RMSE) for the different nonlinear regression models identified above in Equations (6), (7), and (9), the estimated asymptotic D-values, and the estimated CV of the asymptotic D-values for each survival curve. The model described by Equation (6) is referred to as the HSB model in reference to the initials of the authors of the two articles mentioned above in which this model is derived. The RMSEs (Table III) are similar with the exception of those for serotype E, for which the RMSEs for the 1-stage adjusted model are substantially larger than those of the other curves, and for one replicate of the cocktail, for which the RMSE for the 1-stage adjusted model is substantially less than the others. Further, almost all the HSB and all the 1-stage model fitted curves have positive slopes at $t = 0$. Some of these slopes are relatively large and thus, in these cases, there are poor fits for the portions of the survival curves near 0. From Table III it is seen that, within serotypes, the estimated asymptotic D-values of the three models are not too different, with one exception: for one replicate of serotype E, the estimated asymptotic D-value for the HSB (Equation (6)) model was 0.34 minutes, while those for the logistic and the 1-stage adjusted models were 0.51 minutes and 0.60 minutes, respectively. It should be noted that the error CV of this exceptional asymptotic D-value was 145%, reflecting a severe collinearity of the estimated parameters of the particular survival curve. With this one exception, the error CV values are less than about 15%. The exceptional values noted above suggest that the estimated asymptotic D-values obtained using the logistic model, compared over the experiments, are more stable than the estimates obtained using the other models. This can be seen more formally by computing the variances of the pair-wise differences of the logarithms of the asymptotic D-values, and equating these variances to the sum of the unknown individual variances and solving for them. A higher estimated individual variance for one model would imply less stability of estimated asymptotic values relative to those of another model. The estimated variance for the logistic estimates is negative (=−0.008), while those for the 1-stage model and the HSB models are 0.0162 and 0.022, respectively. Fig. 3 presents plots of the observed logarithmic relative reductions and the fitted survival curves using the logistic function described in Equation (7) for the five serotypes and the cocktail.

From Table III, the estimated asymptotic D-values ranged from about 0.3–0.7 minutes. In the AOV model of Equation (8) of the ln(D-value), the between-replicate effect, σ_e, was estimated to be negative for all models, so that the AOV was modified assuming that replicate effect is 0. For this modified AOV model, the between-serotype CVs of the estimated asymptotic D-values are estimated to be approximately 21–22% for all models; the residual CV values are estimated to be about 18–23%; the total CV values (based on the sum of the two variance components) range from about 27–32%, the highest value being associated with the HSB model (Equation (6)). The standard errors of these measurements were determined by the standard jackknife procedure. The standard error of the between-serotype CV using the logistic model is estimated to be 5.8%; for the 1-stage model the standard error is estimated to be 3.3%. If an assumption of normality were made, then confidence

Table III. For the Five Selected Serotypes and the Five-Serotype Composite of *Salmonella* at 60°C, Labeled A–E in Order as Presented in Table I, Comparison of Root Mean Square Errors, Estimated Asymptotic D-Values (min/$\log_{10}$) and CV of These, Derived from Nonlinear Regressions of Natural Log of Relative Reduction Versus Time (Minute)

		Root Mean Square			Error Asymptotic D-Values			CV of Asymptotic D-Values		
Serotype	Replicate	Logistic	HSB	1-stage	logistic	HSB	1-stage	logistic	HSB	1-stage
A	1	0.356	0.359	0.336	0.41	0.41	0.41	10.6	14.9	4.6
A	2	0.339	0.359	0.385	0.35	0.35	0.35	6.7	10.5	4.4
B	1	0.147	0.149	0.168	0.70	0.69	0.67	3.7	5.3	2.6
B	2	0.379	0.379	0.393	0.72	0.72	0.63	4.4	4.4	5.6
C	1	0.640	0.650	0.626	0.47	0.48	0.46	11.0	13.6	6.5
C	2	0.359	0.376	0.395	0.31	0.31	0.32	8.5	14.2	4.8
D	1	0.425	0.435	0.435	0.44	0.44	0.43	6.5	7.9	4.2
D	2	0.300	0.302	0.290	0.66	0.66	0.62	8.7	9.9	5.3
E	1	0.177	0.198	0.278	0.51	0.34	0.60	15.1	145.4	4.4
E	2	0.190	0.242	0.388	0.52	0.49	0.56	4.8	12.9	5.0
All	1	0.200	0.213	0.269	0.63	0.59	0.66	8.4	21.6	4.5
All	2	0.346	0.346	0.204	0.73	0.73	0.61	5.3	5.3	3.4

intervals could be formed using the *t*-distribution with four degrees of freedom.

In an article,[2] estimates of D-values for 17 serotypes of *Salmonella* enteritidis at 57°C and 60°C in liquid egg white product were given. The estimated values of that article[2] are generally lower than the estimated asymptotic D-values estimated above. At 60°C the 17 D-values averaged about 0.33 minutes, ranging from 0.20–0.52 minutes. The CVs for both temperatures were approximately 26%, which corresponds closely to the estimated total CV of the asymptotic D-values given above. From an analysis

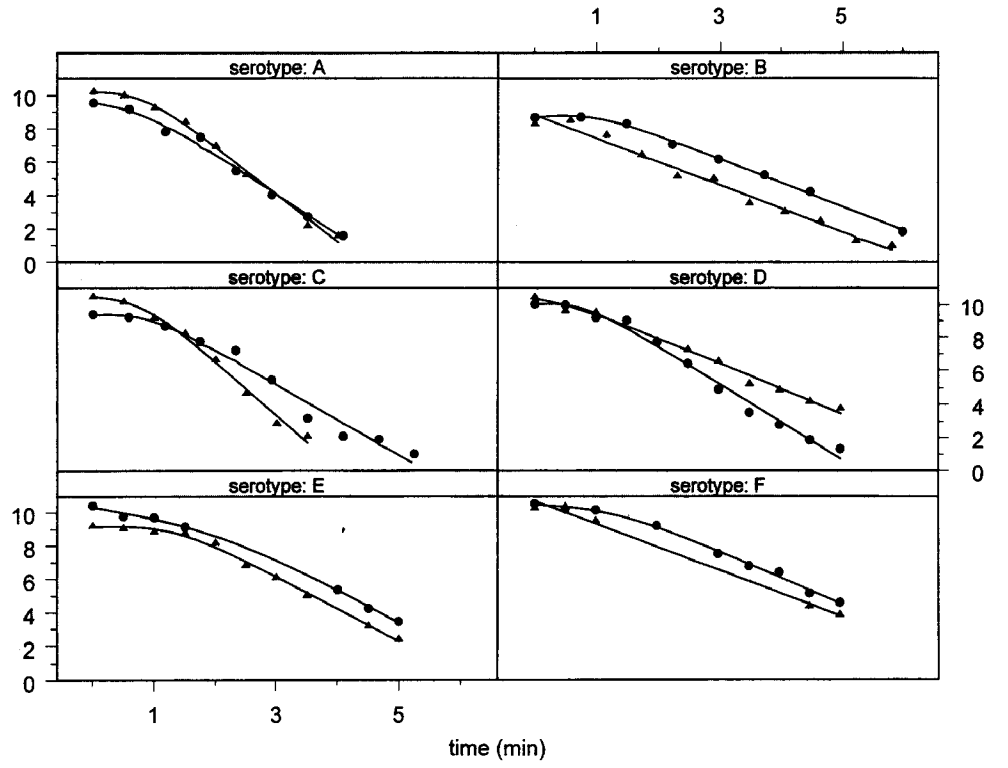

Fig. 3. Observed and fitted survival curves at 60°C for five serotypes of *Salmonella*, labeled A–E in order as presented in Table I, and for the five-serotype cocktail, labeled F. The fitted curves were derived from the equation: $y = -kt - \log_{10}(1 + e^{-b(t-a)}) + \log_{10}(1 + e^{ba})$. Observed points for replicate 1 indicated by ■; replicate 2 by ●.

of variance of the ln(D-values) from the article,[2] there is a statistically significant serotype effect (P-value = 0.04); the between-serotype CV is estimated as 13%, and the CV associated with the residual error, which, since replicate values were not reported, includes the temperature-serotype interaction, is estimated as 22%.

4. DISCUSSION

The following brief discussions center around some difficult issues involved in calculations for estimating the change in *Salmonella* levels for a heating-cooling scenario. Some of the issues would have an impact on the calculations based on the results of the above analysis, and others might have an impact that would enable additional parameters to be modeled if more accurate information were available.

The analysis of variance results given above would be meaningful if the serotypes were randomly selected from a well-defined population of serotypes. It is possible to imagine that the selected serotypes of a study are members of a population consisting of those serotypes that are present, or might be present, in food, so that instead of a finite size population consisting of the selected serotypes, the imaginary population is of infinite size of which the selected serotypes represent a random sample.

4.1. Inactivation

A simple model for predicting D-values in ground chicken, as a function of the fat level, based on results on a cocktail of eight serotypes, is reported in Juneja *et al.*[24] As such, the model, if used in a risk assessment for estimating the expected value of D-values, would introduce a bias because of the significant between-serotype variance calculated above. In an attempt to eliminate this bias and to account for the between-serotype variance in a risk assessment, it might be assumed that the serotypes of a population have parallel thermal death curves: $y_s = y_{s0} + b(T - T_0)$, where s indicates a serotype, the subscript 0 specifies a selected temperature, and b is constant, so that there is a constant z-value for the population. (In the more general case, b is a random variable and the temperature T_0 is selected so that b and y_{s0} are uncorrelated.) Using this restricted model, and assuming a constant fat level of 10%, the thermal death curve for a serotype from a population of serotypes is determined from the equation:

$$\ln(d) = (11.6 - 0.1835T)\ln(10) - g + \varepsilon \qquad (10)$$

where g is an adjustment factor accounting for the bias of the cocktail effect and ε is a random variable with normal distribution, mean, 0 and standard deviation, σ, reflecting the variation of thermal death curves within the population of serotypes and the standard error of g, which is assumed here, for simplicity, not to depend on temperature. For ease of calculation in this example, the coefficients in this model are assumed to be known without error. Thus, specifying g and ε determines the inactivation kinetics for the serotype.

The factor γ and its standard error can be approximated as follows. If we assume that n serotypes of a cocktail are a random sample of serotypes from the population, then the logarithm of the asymptotic kinetic parameter for a composite of n serotypes represents the maximum value of n independent random variables $\{x_j, j = 1, \ldots, n\}$ from a distribution with cumulative distribution function F and density $f = F'$. If $x_{(n)} = \max\{x_j\}$, and $y_{(n)} = F(x_{(n)})$, then it is well known that $y_{(n)}$ is distributed as a beta distribution with parameters n and 1, with central moments μ_j. Thus, the expected value of $y_{(n)}$ is $\mu_1 = n/(n+1)$, and the variance is $\mu_2 = \mu_1(1 - \mu_1)/(n+2)$. Assuming that F is a standard normal distribution with mean = 0 and standard deviation = 1, using the first several terms of the Taylor series expansion, the expected value of $x_{(n)}$, $\hat{z}_{(n)}$, can be approximated as:

$$\hat{z}_{(n)} \approx z_{(n)} + z_{(n)}\mu_2 / \left(2f^2(z_{(n)})\right) \qquad (11)$$

where $z_{(n)} = F^{-1}(\mu_1)$, and the standard error of $\hat{z}_{(n)}$, $s_{(n)}$, can be approximated as:

$$s_{(n)} \approx \mu_2^{0.5} / f\left(\hat{z}_{(n)}\right) \qquad (12)$$

The assumption of normality is used in deriving Equation (11), where the derivative of $f(x) = -xf(x)$.

For an estimated D-value, $D_{(n)}$, of a cocktail of n serotypes, let $v_{(n)} = \ln(D_{(n)})$, and $se(v_{(n)})$ equal the standard error of $v_{(n)}$, estimated with sufficient degrees of freedom so that a normal approximation to the distribution of $v_{(n)}$, conditional on the selected serotypes, can be considered sufficient. For simplicity in these calculations, as mentioned above, it is assumed that the error due to regression is small and does not contribute much to the total standard error of the estimate, so that $se(v_{(n)})$ can be approximated by a constant. Assume that the distribution of ln(d), where d is a random variable, representing D-values, from the population of serotypes, is normal with mean, m, and standard deviation of η. The mean m is estimated as:

$$\hat{m} = v_{(n)} - \eta\hat{z}_{(n)} \qquad (13)$$

with estimated standard error of

$$stderr(\hat{m}) = \sqrt{se^2\left(v_{(n)}\right) + \eta^2 s_{(n)}^2}. \qquad (14)$$

Thus, the factor g is equal to $\eta\hat{z}_{(n)}$. The thermal death curves, expressing the values of the natural log of D with temperature, can be generated from a normal distribution with mean equal to $\hat{m}$, from Equation (13), and standard deviation, s, as an estimate of σ, where

$$s = \sqrt{se^2\left(v_{(n)}\right) + \eta^2\left(1 + s_{(n)}^2\right)}. \qquad (15)$$

For our example, $n = 8$, $\hat{z}_{(8)} = 1.39$, and suppose $\eta = 0.20$ (corresponding to a between-serotype CV of about 20%). The value, $v_{(8)}$, is derived from Equation (10) as: $(11.6 - 0.1835T)2.303$; $g = 0.28$; the mean of the population is estimated: $\hat{m} = v_{(8)} - 0.28$; and $s_{(8)} = 0.65$, so that the standard deviation s, estimating σ, used in a risk assessment would be $(se^2(v_{(8)}) + 0.057)^{0.5}$. If, as above for *Salmonella*, the repeatability (between replicate CV) were estimated to be about 20% with two replicates, so that $se^2(v_{(8)}) = 0.04/2 = 0.02$, then the standard deviation, s, would be 0.277 ln(minutes). Thus, ε, from Equation (8), is determined randomly by generating a normal variate with mean equal to 0 and standard deviation equal to 0.277 ln(minutes). In common log units, this would be 0.120 $\log_{10}$(min). Thus, a 99% probability interval for D-values would span a range of 4, but the expected value would be about 25% less than the value estimated from the cocktail model (Equation (10)) ignoring the bias correction term, g.

4.2. Growth

The analysis of the growth kinetic parameters suggested that between-serotype effects would be small, relative to the experimental variability. Consequently, serotype effects, if not included in a risk assessment for *Salmonella* spp, would, for the most part, not create a significant bias relative to other sources of error.

However, when modeling growth in any medium, an account is needed of the inherent random variation of the growth or division of cells when there are a small number of cells, and of the decreasing growth rates when there are large number of cells as the cell population approaches stationary phase. For the latter, it has been assumed that there is a fixed maximum level, M, that can be reached and that as levels approach this maximum level, the growth rate decreases. The magnitude of M could be a function of many variables, including the medium and the competitive flora present. For example, the magnitudes of M and the exponential growth rates seen in studies for *E. Coli* O157:H7 have been observed to be substantially less in meat that has not been irradiated than in meat that has been irradiated or in broth.[25] Recent work[26] demonstrates effects on exponential growth rates, lag phase duration, and perhaps M, in *E coli* O157:H7 due to agitation conditions (shaking vs. nonshaking) and initial density in inoculated broth samples at low temperatures. Thus, a good deal of research is needed to determine the actual growth kinetics in different media and conditions.

Equation (1) describes the expected value of growth, or the expected growth curve. But an actual growth curve for a given realization could be substantially different from the one predicted from Equation (1), even if the assumptions used for deriving Equation (1) and the estimated parameter values were true, due to the inherent variability of cell growth. The time that it takes the original O-cells to leave the lag phase and the time it takes D-cells to divide are random variables. For a risk assessment, assumptions concerning the distributions of these random variables are needed in an attempt to capture fully the range of possible risks. Equations that account for this variability can be derived by employing the theory of stochastic processes that was developed in the middle 20th century.[27] The assumptions used in deriving equations are: (1) the probability distribution of the number of cells at a given time $t > \tau$ depends only on the number of cells at time τ and not on any historical events that took place before τ; (2) episodic or instantaneous events of increases in the number of cells by more than 1 have a probability of virtually zero—a Poisson-like assumption that is used for simple linear birth or death processes;[27] and (3) events of cells leaving lag or dividing are mutually independent at any time. With regard to the lag phase duration of cells, and thus the quantity $\lambda(t)$, the first assumption might be problematic because it has been reported that the lag phase duration of cells depends significantly on the historical experience of the cells, at least when there is a sudden and substantial environmental change.[28] The second assumption excludes twins or more offspring resulting, that is, cells do not divide into more than two parts.

With these assumptions the probability distribution of the number of cells at time t under general conditions of changing temperatures can be derived. The parameters of Equation (1), μ and λ, are expressed as functions of time, described by some function such as the Ratkowsky function.[15] A short derivation of the probability distribution of the number of cells as a

function of time is given in the Appendix, using generation functions, where it is assumed that the length of time for an O-cell to become a D-cell is distributed with cdf H. The derived formula for $(E(r(t)))$ is:

$$E(r(t)) = e^{-\gamma(t)} + \int_0^t e^{v(t)-v(\tau)}\, dH(\tau) \qquad (16)$$

where $\gamma(t) = \int_0^t \lambda(\tau)\, d\tau$ and $v(t) = \int_0^t \mu(\tau)\, d\tau$. When H is an exponential distribution with parameter λ, and μ is constant, then Equation (16) reduces to Equation (1). Baranyi[13] developed Equation (1) using a deterministic model. His development can be extended slightly by letting the kinetic parameters be functions of time. Let $m_A(t)$, $A = O$ or D, represent the number of cells at time t. The following set of differential equations:

$$\begin{aligned} \dot{m}_O(t) &= -\lambda(t)m_O(t) \\ \dot{m}_D(t) &= \lambda(t)m_O(t) + \mu(t)m_D(t) \end{aligned} \qquad (17)$$

with boundary conditions, $m_0(0) = N_0$ and $m_D(0) = 0$, are derived reflecting the assumptions: (1) the population of O-cells follows a first-order kinetic decay with parameter $\lambda(t)$; and (2) the D-cells increase due to two sources: (a) the deaths of O-cells, and (b) the births of D-cells, described kinetically by parameter $\mu(t)$. The solution to the differential equations of Equation (17) leads to an expression for the predicted relative growth, $r(t)$,

$$Pred(r(t)) = e^{-\gamma(t)} + e^{v(t)} \int_0^t \lambda(\tau) e^{-(\gamma(\tau)+v(\tau))}\, d\tau \qquad (18)$$

which is the same as the expected value given of Equation (16). The differential equations:

$$\begin{aligned} \dot{m}_O(t) &= -\lambda(t)m_O(t) \\ \dot{m}_D(t) &= \lambda(t)m_O(t) + \mu(t)m_D(t)(1 - m_D(t)/M) \end{aligned} \qquad (19)$$

with boundary conditions, $m_0(0) = N_0$ and $m_D(0) = 0$, which describe the process are similar to those of Equation (17), except for the inclusion of a term (logistic) that accounts for the decreasing growth rate when the number of cells becomes large. Fig. 4 presents the growth curve derived from the above

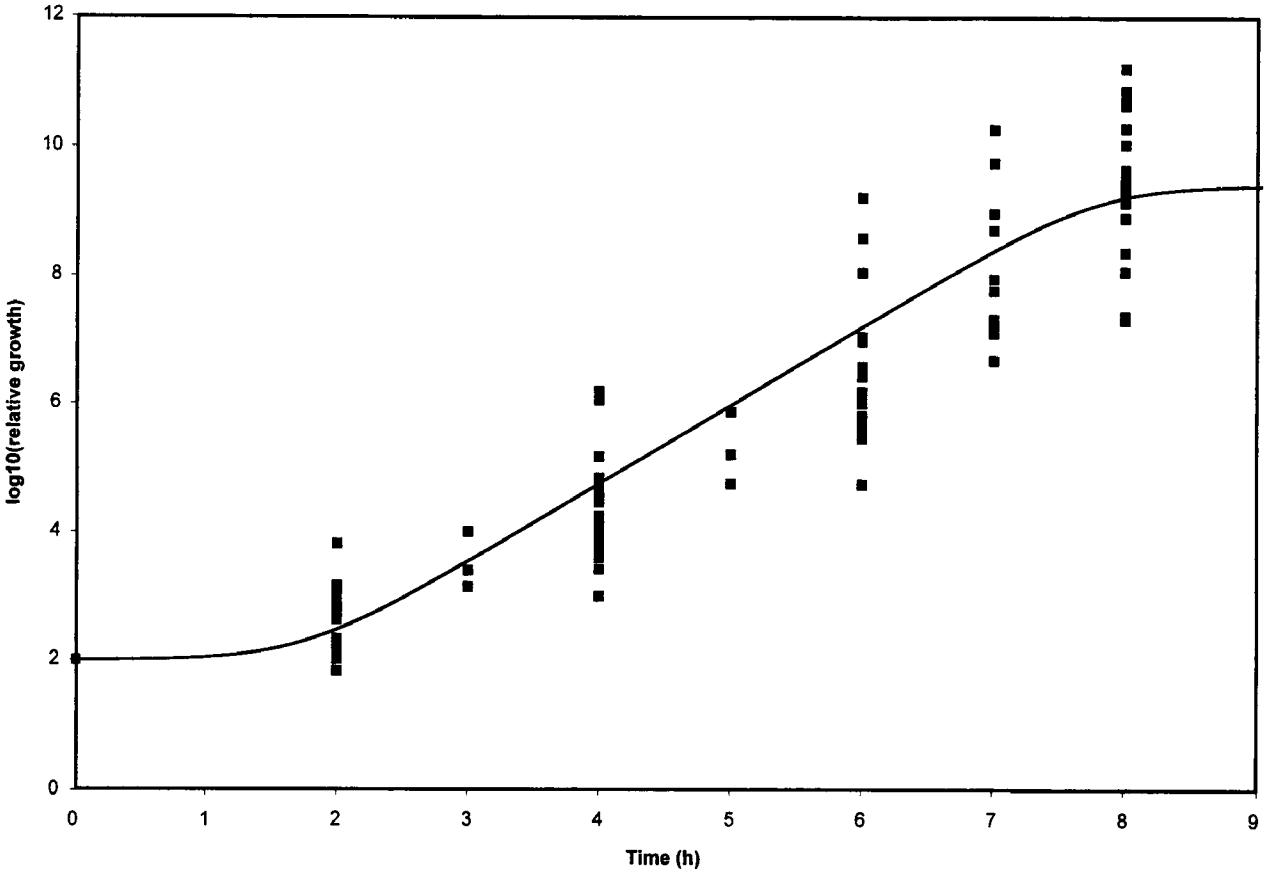

Fig. 4. "Average" fitted growth curve at 37°C compared with observed $\log_{10}$ relative growth for all 10 serotypes of *Salmonella*.

equations, assuming $\lambda = 0.02$, $\mu = 2.83$, $M = 9.5$, and $N_0 = 100$. The displayed data points are the observed $\log_{10}$ relative growths plus 2 $\log_{10}$ at 37°C for all the experiments of the individual serotypes. As is seen, there is a good deal of variability around the fitted growth curve; however, the curve described by the assumed values of the parameters seems to provide a reasonable average fit for the observed data. From Equation (A5), assuming one O-cell ($N_0 = 1$), the probability that there would be still be only one cell at the end of five hours is about 91%. The expected value, from Equations (16) or (18), of the number of cells at five hours is about 10^4. If there were 10 cells initially, then there is about a 40% probability that there would be still 10 cells at five hours, even though the expected number of cells would be about 10^5. Even with 50 cells, there is about a 1% chance that there would not be an increase in the number of cells after five hours. Thus, when there are, initially, a small number of cells, the expected value of the number of cells would be a poor "representative" value for the actual number of cells. This also can be understood by computing, from Equation (A4), CV $\approx$ 1250% for $N_0 = 1$, and CV $\approx$ 395% for $N_0 = 10$. If this interpretation is valid, even in approximation, these calculations show that, for small to moderate numbers of cells, it is critical for risk assessments to account for the variation in the increase of the number of cells by using probability distributions for describing the growth possibilities.

5. CONCLUSIONS

This article explores some of the predictive microbiology issues that impact estimates of microbial risk. Many factors affect the growth and survivability of pathogens; there is extensive research on these issues, and more is needed. However, the issues discussed in this article involve mathematical and probabilistic issues that could affect the accuracy of predictions of possible numbers of cells in portions of food, subjected to given situations or processes.

In this article, the between-serotype of *Salmonella* variation of kinetic parameters describing growth and inactivation were examined. For growth there did not appear to be a significant between-serotype effect. Generally, the *Salmonella* survival curves were concave, with asymptotic D-values. For the five serotypes of *Salmonella* studied, the between-serotype CV of the asymptotic D-values at 60°C was estimated to be about 20%, with standard error of about 3% or 6% depending on the model used.

The expected growth or inactivation rates derived from data using cocktails are biased, reflecting the characteristics of the fastest growing or most heat resistant serotype of the cocktail. In the discussions presented in this article, an adjustment to decrease this bias and to account for between-serotype variation is offered when information is available on the between-serotype variances within a population of serotypes. Information concerning the interaction of serotypes over the range of conditions is needed to assure that unbiased estimates are obtained.

For growth of a small number of cells, a better understanding of the probability distribution of phases of cell growth among cells in a population is needed. The equations given in this article indicate, with a small number of cells, that, simultaneously, there could be significant probabilities of no growth or a very large amount of growth, so that the expected value of the growth curve would not be a good representative value of the possible growth for use in a risk assessment. Microscopic investigation of cell development is needed to clarify the situation more accurately.

AUTHOR NOTE

Mention of brand or firm name does not constitute an endorsement by the U.S. Department of Agriculture above others of a similar nature not mentioned.

APPENDIX

Stochastic Growth

It is assumed that there is a population of cells, referred to as O-cells, in stationary phase. At time $t = 0$, the environment has somehow changed, permitting the cells to grow. The O-cells must pass through a "lag" phase before growth is possible. When the cell completes its lag phase, the cell is referred to as a D-cell. It is assumed that when a D-cell divides it produces two new D-cells, and that D-cells grow according to a simple linear birth process. Also, it is assumed that all the passage events are mutually independent. Thus, equations describing the distribution of the number of cells from the growth of N cells can be derived from equations describing the growth from a single cell.

Let $v(t,s) = \int_s^t \mu(\tau)\, d\tau$ where $\mu(t)$ is the exponential growth rate of D-cells, assumed to be a function of time, t. If t_0 is the time an O-cell becomes a D-cell,

then the distribution of the increase in the number of D-cells, $I(t)$, associated with the one D-cell at time $t > t_0$, is the geometric distribution[14] with generating function:

$$g_1(s, t - t_0) = \frac{e^{-v(t, t_0)}}{1 - \left(1 - e^{-v(t, t_0)}\right)s}. \quad \text{(A1)}$$

Thus, conditional on the time, t_0, the generating function, $F(s, t \mid t_0)$, for the number of cells, from a given original cell, is:

$$F(s, t \mid t_0) = \iota(t < t_0)s + \iota(t \geq t_0)[sg_1(s, t - t_0)] \quad \text{(A2)}$$

where $\iota(\,)$ is equal to 1 if the argument is true, otherwise it is equal to 0. Let $H(t) = \text{Prob}(t_0 < t) = 1 - e^{-\gamma(t)}$ be the cdf of the transition time, where $\gamma(t) = \int_0^t \lambda(\tau)\, d\tau$. The unconditional generating function of the number of cells $F(s, t)$ is:

$$F(s, t) = se^{-\gamma(t)} + \int_0^t sg(s, t - \tau)\, dH(\tau) \quad \text{(A3)}$$

where $dH(\tau) = \lambda(\tau)e^{-v(\tau)}\, d\tau$.

The above equation is similar to one used by Barayani and Pin,[22:Eq. 10] except their equation was describing the expected value rather than the generating function and assumed that μ was constant. Equation (A3) describes the generating function for the number of cells given one O-cell. It is assumed that the times in lag phase and of cell division are independent, thus the expected value and the variance of the relative growth, $r(t) = N(t)/N_0$, where $N(t)$ is the number of cells at time t, and N_0 is the number of cells at $t = 0$, can be obtained using the properties of generating functions. The derived equations are:

$$\begin{aligned} E(r(t)) &= e^{-\gamma(t)} + \int_0^t e^{v(t,\tau)}\, dH(\tau) \\ N_0 var(r(t)) &= 2\int_0^t e^{2v(t,\tau)} - e^{v(t,\tau)}\, dH(\tau) \\ &\quad + E(r(t))(1 - E(r(t))). \end{aligned} \quad \text{(A4)}$$

From Equations (A1) and (A3), when $N_0 = 1$, the probability of j organisms are:

$$\begin{aligned} p_1(t) &= e^{-\gamma(t)} + \int_0^t e^{-v(t,\tau)}\, dH(\tau) \\ p_j(t) &= \int_0^t e^{-v(t,\tau)}\left(1 - e^{-v(t,\tau)}\right)^{j-1} dH(\tau), \quad j > 1. \end{aligned} \quad \text{(A5)}$$

When $N_0 > 1$, the generating function of the number of organisms is $(F(s, t))^{N_0}$, so that for small N_0 the probabilities of the number of organisms can be computed, given the p_js. For large N_0, approximations using extreme value or log-normal distribution could be used.[14]

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